UCSB

Immunocapture & Isolation of BrdU-Labelled DNA

(adapted by Dad Roux-Michollet from Kathleen Treseder at UC Irvine)

Equipment

- Microcentrifuge (high speed = 15 000 rpm)
- Hybridization oven/incubator at room temp (horizontal rotation)
- Dynal MPC-S Magnetic Particle Concentrator (Dynal/Invitrogen #120-20D)
- Heat block (100°C)
- Two ice buckets with ice (thawing and dry ice/ethanol bath)
- Dry ice (4 lbs.) → Bio. Store
- 70% ethanol for bath (1.5 l): 100% ethanol + d.i. water
- Timer

Reagents

- Dynabeads Sheep Anti-Mouse IgG (Dynal/Invitrogen #110-31)
- Anti-BrdU antibody (Roche #11170376001)
- 1.7 mM BrdU solution
- Herring sperm DNA, 10 $mg.ml^{-1}$, 10 mg (Promega #D1811 also available from Fisher)
- PBS solution
- PBS-BSA solution
- 0.22 µm filter sterilization equipment

1.7 mM BrdU Solution

- Mix 17 µl of **100 mM Brdu** solution and 983 µl **PBS-BSA**

(100 mM BrdU = 0.307 g BrdU + 10 ml H_2O)

- Store at -20°C for up to 6 months (140 µl aliquots)

Phosphate-Buffered Saline (PBS)

8.0 g Sodium Chloride (NaCl)
0.2 g Potassium Chloride (KCl)
1.44 g Sodium Phosphate Dibasic (Na_2HPO_4)
0.24 g Potassium Phosphate Monobasic (KH_2PO_4)

- Dissolve in 800 ml deionised water
- Adjust to pH 7.4 with HCl or KOH
- Bring deionised water to the final volume (1 l)
- Autoclave to sterilize and store at room temperature

Phosphate-Buffered Saline-Bovine Serum Albumin (PBS-BSA)

- Mix 50 ml PBS and 0.05 g non-acetylated BSA (Sigma #A7906)

- Filter-sterilize (0.22 µm) and store at 4°C for up to 3 months

(**need about 1 mL per sample*)

Anti-BrdU Antibody

- Dilute antibody 1:10 in **PBS** → 100 µl antibody in 900 µl PBS
- Aliquot into small tubes for storage (**need 2 µl per sample*)
- Store at -20°C until original expiration date (label aliquots with expiration date on the container)

(****Avoid freeze-thaw → very sensitive***)

Herring Sperm DNA Working Solution (1.25 mg mL^{-1})

- Vortex herring sperm DNA
- Mix in 1.5 ml microcentrifuge tube
  - 100 µl herring sperm DNA
  - 250 µl cold 100% ethanol (**EtOH chilled at -20°*)
  - 10 µl **3 M sodium acetate**
- Incubate the mixture at -20°C (walk-in freezer) for 10 min
- Centrifuge at room temperature for 30 s at 3000 rpm (**should form a loose white pellet*)
- Remove and discard supernatant (pipette or decant), then drain tube upside-down on KimWipe
- Add 250 µl chilled 70% EtOH (diluted with sterile water), mix gently
- Centrifuge at room temperature for 30 s at 3000 rpm
- Remove and discard supernatant (decant or pipette off liquid) then leave tube open for 10 min at room temperature to remove residual liquid
- Add 800 µl PBS and vortex for 3 min
- Incubate at 37°C for 3 min or until DNA goes completely into solution (occasional vortexing during the incubation can facilitate this process)
- Aliquot the DNA solution (around 7 x 110 µl), then store at -20°C for up to 6 months

Preparation

- **Heat Block** → set to 100°C
- Thaw on ice:
  - α-BrdU antibody
  - Herring sperm DNA
  - Extracted sample DNA containing BrdU-labeled DNA
- **Dry Ice-Ethanol Bath** (*~ 30 minutes*)
  - Break about 4 lbs. of dry ice into smaller pieces using some metal tool
  - Gently add about 500 mL 70% ethanol to dry ice into ice bucket
  - Keep adding ice and ethanol (1 l), mixing until bath becomes thick & gooey (**tubes should float*)
  - Bath will continue to bubble gently (*some bubbling and pieces of dry ice floating around is fine. You will probably have to add more dry ice/ethanol and then let settle for about 5 min right before you need to use the bath, as the mixture does sublimate quickly*)
- **Hyrbidization incubator** → set to room temperature

Procedure

Prepare Tubes

- For each sample, label 3 sets of 1.5 mL tubes with sample identification:
  - HS for herring sperm
  - Sample for sample DNA
  - BrdU DNA for final eluted DNA (use more detailed label for this tube)
- Beads for DynaBeads (1 tube for 3 samples)

Denature and Flash-Freeze HS DNA

Time is critical in this step* → ***move quickly*

- Add **18 µL** of herring sperm DNA solution to the first set of tubes
- Incubate HS tubes in heat block (100°C) **for exactly 1 minute**
- Transfer tubes in dry ice bath bubbling for **30-50 seconds** to flash freeze

(**make sure the liquid in the tubes is submerged in the bath, and then frozen*)

- Thaw tubes completely at room temperature (less than **3 min**)
- Centrifuge on quick spin for about **5 s**

Formation of HS DNA/α-BrdU Antibody Complex

- Add **2 µL** of **α-BrdU antibody** to denatured HS DNA → 20 µL total
- Pipette gently up/down to mix → Avoid creating bubbles and try to keep liquid at bottom of tube (spit tip into tube then change volume on pipette)
- Incubate at room temperature for **40-50 min** (dark + occasional mixing)

(**Check ice bath → add more dry ice and ethanol if necessary*)

Preparation of DynaBeads

Initial Preparation

- Vortex beads to mix
- Add **25 µL** of beads into 1.5 mL tubes (1 tube for every 3 samples)
- Centrifuge at top speed for 2 seconds (about 16 seconds total)
- Place tubes in MPC and pipette out the liquid → avoid brown beads on back

Wash DynaBeads

3x
- Remove MPC and add **200 µL PBS-BSA** to each tube
- Flick tube gently to resuspend beads then put back in MPC
- Remove liquid at the bottom of tubes with pipette (**Avoid dynabeads*)

- After final wash → centrifuge tubes for 2 seconds at top speed (about 16s) then put back in MPC to remove any remaining liquid with 10 µL pipette

Resuspend DynaBeads

- Remove MPC, add **25 µL** of PBS-BSA to each tube, pipette gently to resuspend (avoid bubbles), and put on ice

Prepare Sample DNA for Immunocapture

**Do this after HS DNA-antibody complex has been incubating for about 20-30 min*

- Add **20 µL sample DNA** to second set of tubes (good concentration ~ 20 ng μL^{-1})
  - * *If concentration is high → dilute in PBS*
- Pipette gently to mix (avoid bubbles) then put on ice until HS-antibody incubation is almost done

Denature and Flash-Freeze Sample DNA

- Repeat denaturing procedure on sample DNA

(**Turn off heat block and discard ice bath after this step → can also put reagents away*)

Formation of DNA/α-BrdU Antibody Complex

- Add denatured soil sample DNA to HS-antibody complex tube (40 µL total)
  **Avoid any condensation droplets around top of tube*
- Pipette gently once or twice to mix (avoid bubbles)
  (**Do not spin down*)
- Incubate at room temperature in dark for **30 min**

Formation of DNA/α-BrdU Antibody/DynaBead Complex

- Tap beads to resuspend, add **6.26 µL** washed beads to each sample
- Pipette gently to mix
- Put 3 microcentrifuge tubes inside a 50 mL Falcon tubes (stabilize with aluminium foil and KimWipes)
- Place in hybridization oven: wrap Falcon in paper and clip directly into oven rack
- Rotate tubes (speed around **12**) at room temperature for **30-40 minutes** (horizontal position)

(**The reason for using a horizontal incubator is to constantly, but gently and slowly agitate the tubes so that the heavy dynabeads stay suspended*)

Wash DNA/∝-BrdU Antibody/DynaBead Complex

* *Total of 8 washes with 100 µL PBS-BSA → use more if getting unlabelled DNA contamination*

- Centrifuge at top speed for 2 seconds
- Place tubes in MPC → remove and discard liquid using 200 µL pipette

4x
- Remove MPC, add 100 µL PBS-BSA (using repeat pipette) and tap gently to resuspend
- Replace MPC and remove liquid

- Centrifuge at top speed for 2 seconds
- Place tubes in MPC → remove and discard liquid with 10 µL pipette

3x
- Remove MPC, add 100 µL PBS-BSA (using repeat pipette) and tap gently to resuspend
- Replace MPC and remove liquid

- Centrifuge at top speed for 2 seconds
- Place tubes in MPC → remove and discard liquid with 10 µL pipette
- Remove MPC, add 100 µL PBS-BSA and tap gently to resuspend
- Replace MPC and remove liquid
- Centrifuge at top speed for 2 seconds
- Place tubes in MPC → remove and discard liquid with 10 µL pipette

Release Labelled DNA from the Complex (Remove the tubes from the MPC)

- Add 20 µL of 1.7 mM BrdU to tubes and tap gently to resuspend the beads
- Rotate in hybridisation oven as above for **30-40 min**

(**Because the α-BrdU antibody has a greater affinity for pure BrdU molecules, the labeled DNA will be released from the α-BrdU/dynabead complex when the antibodies preferentially bind to the pure BrdU*)

Collect BrdU-Labelled DNA (Avoiding the brown dynabeads)

- Remove tubes from hybridisation oven and centrifuge at top speed for 2 seconds.
- Place tubes in MPC and transfer liquid from bottom of tube to last set of labelled tubes
  (**Contains the sample DNA → about 20 µL total*)
- Store at -20°C (short term) or -80°C (long term)
- May want to make small aliquots to avoid freeze-thaw damage during PCR, etc.

Immunocapture/Isolation of BrdU-labeled DNA

The following procedure is designed to separate BrdU-labeled DNA from unlabeled DNA that has been extracted from environmental samples. We recommend purifying **total extracted DNA** from environmental samples that have been incubated with BrdU (see associated protocol on BrdU incubation) by using the MoBio DNA extraction kits.

This procedure is very sensitive – don't be surprised if you can't get it to work. The most critical parts of the process are the denature/freeze steps and the wash steps. The denaturing must be done at 100 C using a heat block or bath that will stay stable at 100. The flash freezing must be done with ethanol/dry ice. The wash steps are necessary to decrease the amount of unlabeled DNA contamination in your final DNA yield. It is highly recommended that you run at least one no-BrdU (water only) incubated control sample in parallel with your samples in order to check for the relative amount of unlabeled DNA that will inevitably end up in your final DNA. We have found that this can be minimized to about 10% of your final DNA if you follow this procedure. (Other labs have indicated similar levels of unlabeled DNA contamination after immunocapture. See refs for BrdU incubation protocol.).

HS DNA/α-BrdU Antibody Complex is necessary in order to bind any antibody molecules that are not specific enough to bind to BrdU-labeled DNA. Because specific antibodies are created in live mammals, some of the antibody molecules will not be very good at distinguishing between labeled and non-labeled DNA. Herring sperm DNA is used to bind these "non-specific" molecules before they are exposed to the sample DNA, thus theoretically eliminating the possibility that they will bind non-labeled DNA within the sample.)

The final DNA must be released from the antibody complex by addition of a 1.7mM BrdU in PBS-BSA solution - thus the final DNA is dissolved in PBS-BSA, which contains protein. This makes it impossible to estimate DNA concentrations using a spec, but the final DNA concentration is usually so low that you can't see the DNA on a gel either. The only way to check that you have DNA in your final sample is to PCR your samples, including the "no-BrdU" control, with the appropriate primers. Additionally, PCRs from immunocaptured DNA are particularly difficult because of low template concentration and BSA (potential PCR inhibitor at high concentrations) in the template DNA. If you typically use BSA in your PCRs, you may have to decrease the amount of BSA in your PCR reactions for immunocaptured DNA. I highly recommend that you perform a few test runs of the procedure from BrdU incubation up through PCR so that you can be sure the entire process will work for your particular environmental sample – all soil, sediment, litter, etc, have different chemistries, which can affect every step of the BrdU process up through PCR. Also, as PCRs can typically be difficult with immunocaptured DNA these test runs are recommended to create a sample of immunocaptured DNA that you can use to optimize your PCRs. We use total DNA (before immunocapture) from one of the samples of interest as a PCR positive control. Also, when optimizing the PCR, always check the amplification of your "no-BrdU" control. A good number of cycles to shoot for in your final PCRs is that which gives you a good enough band in your BrdU-added samples and either no band or very faint band in your no-BrdU control.